CHAPTER ONE

INTRODUCTION

1.INTRODUCTION

1.1. **Overview:**

brucellosis is a serious disease of livestock that has significant animal health, public health, and international trade consequences. Considering the damage done by the infection in animals decreased milk production, weight loss, loss of youngs, infertility and lameness. This disease is a

formidable threat to livestock. The fact that this disease can spread rapidly and be transmitted to humans makes them all the more serious.

Brucellosis is world-wide in distribution and has known historically as undulant fever, Bangs disease , Gibraltar fever, Mediterranean fever, and Malta fever. The organism was first isolated in 1887 by Sir David Bruce, who recovered a suspected organism from splenic culture of British soldier dying of Malta fever (Koneman *et al.,* 2006*).*

The brucellae are obligate parasites of animals and human and characteristically located interacellularly. They are relatively inactive metabolically, *Brucella melitensis* typically infect goats; *Brucella suis*, swine; *Brucella abortus,* cattle; and *Brucella canis*, dogs. Other species are found only in animals. Although named as species, DNA relatedness studies have shown there is only one species in the genus, *Brucella melitensis,_* with multiple biovars. The disease in humans brucellosis (undulant fever or Malta fever), is characterized by an acute bacteremic phase followed by a chronic stage that may extend over many years and may involve many tissues. (Brooks *et al.,* 2007).

Brucellosis is a widespread zoonosis mainly transmitted from cattle, sheep, goats, pigs and camels through direct contact with blood, placenta, foetuses or uterine secretions, or through consumption of contaminated raw animal

products (especially unpasteurized milk and soft cheese) (WHO, 2005).

Main modes of transmission includes ingestion, direct contact through breaks in the skin and airborne infection (laboratories and abattoirs), primarily affecting consumers of raw milk and derivatives, farmers, butchers, veterinarians and laboratory personnel. The incubation period is highly variable, usually 2-4 weeks, can be 1 week to 2 months or longer (WHO, 2005).

Brucellae are intracellular organisms infecting reticuloendothelial cells of spleen, liver, kidneys and bone marrow. From this site, the bacteria pass into blood. The disease is characterized by fever which may be continuous, intermittent, undulating or irregular. Acute infection may resemble severe influenza, headache, sweating and generalized pains associated with fatigue and depression. Untreated infection can become chronic with musculoskeletal symptoms (back pain, arthritis, arthralgia). Heart valves involved lead to endocarditic (Cheesbrough, 1991).

The diagnosis of brucellosis is primarily dependent on clinical suspicion allied with the taking of an adequate history of possible exposure - including during travel. Presentation can, however, be highly atypical and focal lesions may present decades after exposure. Unequivocal diagnosis requires isolation of the organism. Blood culture is the method of

choice but specimens need to be obtained early in the disease and cultures may need to be incubated for up to four weeks. Even so, failure to growin the organism is common, especially in cases of *B abortus* infection, and isolation rates of only 20-50% are reported even from experienced laboratories. Modern commercial systems are hampered by the small amount of CO_2 produced during growth. Culture from bone marrow and from presenting foci may be successful. Presumptive identification of cultures from morphology and slide agglutination with specific antiserum should be followed by further work in a reference facility. Molecular techniques for typing are being developed (Alton and Forsyth, 2008).

1.2. **Rationale:**

Brucellosis is a zoontic disease with worldwide distribution. Transsimationt of brucellosis by direct contact with infected animal and by raw ingestion of milk or unpasteurized milk product.

Brucella has cross reactive microorganisms in serological tests such as *Yersinia interocolitica o:9* , *Escherichia coli o:157* and *Salmonella urbana,* some types of *Vibrio ,pasteurella. (Alton,G et al,. 1975).*

Brucellosis appear in females more than males and cause many manifestation special in reproductive system. (Hassan.R, 2013)

Brucellosis from cases study cause disrupt in menstrual cycle , illness with period, abortion, and infertility. Severe infections of central nervous system, lining of the heart, joint pains, orchitis, meningitis and fatigue.

The infection causes more than 500,000 cases per year world-wide

(WHO, 2005).

1.3. Objectives:-

1.3.1 General objective

TO determine seroprevalance of brucellosis among reproductive women

1.3.2 Specific objective

1.To detect acute Brucellosis in reproductive women by slide agglutination test and confirmed by enzyme linked Immunoassorbent assay(ELISA).

2.confirmed the agglutination test by ELISA test.

3.To investigate the prevalence between different occupation groups

CHAPTER TWO
LITERATURE REVIEW

LITERATURE REVIEW

2.1. History

The history of brucellosis does not begin with the isolation and identification of *Brucella melitensis* (*Micrococcus melitensis*) in the 1880s. Many historical accounts of diseases before this time could actually be describing brucellosis including abortion epidemics in animals and fever in humans. Other than biblical references to animal abortions, one of the earliest recorded descriptions of brucellosis was made by Marston in 1859 ,He wrote of an illness, including of his own, which differed from typhoid fever. There are other recordings of how, what is now believed to have been brucellosis, disease affected the Crimean War and sailors aboard ships. Brucellosis then called Mediterranean fever and was a debilitating chronic illness with the complication of rheumatism for which many Royal Naval seaman were invalidated each year

Captain David Bruce was sent to Malta and, with several others, conducted research from 1884. He isolated an agent called *Micrococcus melitensis* from human spleens (Rahman. *et al,* 2006).

Brucella is named after Sir David Bruce who, in 1886, first isolated the organism from the spleen of a soldier at *post-mortem* with what was then called Malta fever. The genus *Brucella* currently contains six nomen species: *B. melitensis*, *B. abortus, B.canis, B.ovis, B.suis and B.neotomae,* which vary in their ability to infect host animals.

B. abortus primarily infects cattle but is transmitted to buffaloes, camels, deer, dogs, horses, sheep and man.

B. melitensis causes a highly contagious disease in sheep and goats although cattle can be infected. It is the most important species in human infection.

B. suis covers a wider host range than most other *Brucella* species.

This species has 5 biovars, biovars 1 and 3 infect swine primarily, biovar 2 causes infection in European wild hares, biovar 4 is responsible for infection in reindeer and wild caribou and Biovar 5 was isolated from rodents in the USSR. All biovars can be transmitted to man with the possible exception of biovar 2.

B.canis causes epididymo-orchitis in the male dog and abortion and metritis in the bitch. It has not been reported in other animal species except man.

B.ovis is responsible for epididymitis in rams and occasionally abortion in ewes, but does not infect other animals or man. Goats are susceptible to the disease by experimental infection.

B.neotomae is only known to infect the desert wood rat under natural conditions, and no other cases have been reported (Stack and MacMillan, 2003).

To date, six terrestrial and three marine Brucella species have been recognized: *B.melitensis* (preferred hosts are goats, sheep, camels), *B.abortus* (cattle, buffalo), *B.suis*

(swine and a range of wild animals), *B.canis* (dogs), *B.ovis* (sheep), *B.neotomae* (desert and wood rats) and *B.delphini, B.pinnipediae* and *B.cetaceae* from marine mammals (e.g. seals, whales, dolphins). The first four species can infect humans, with *B.melitensis*, *B. abortus* and *B.suis* causing the most disease in both humans and animals. Brucella spp. associated with marine animals have been reported to cause disease in humans (George, 2010).

2.2. Distribution of disease in Sudan

In Sudan, the disease was suspected as early as 1904. Simposon (1908) reported 20 clinically diagnosed cases in the Blue Nile and Kassala provinces. Bennet (1943) isolated *B.abortus* for the first time from a dairy herd in Khartoum. Hasseb (1950) was the first to confirm a case of human Brucellosis. Hasseb (1950) and Daffala (1962) stated that the disease was diagnosed in all provinces except Bahr El Gazal, In the Southern Sudan up to 1955 (Hassan, 2013).

Brucellosis in Sudan was first reported from human cases as early as 1904. The organism was also isolates from camels in Butana area *B.abortus* was first isolated from a dairy farm in Khartoum, while *B.melitensis* was isolated from goat's milk among British residents in the El Gezira area. Many investigators reported the disease from different parts of Sudan (Hassan, 2013).

2.3. Rote of Transsimationt

Humans are generally infected with brucellosis in one of three ways: Breathing in the bacteria that cause brucellosis (inhalation) Eating undercooked meat or consuming unpasteurized/raw dairy products Bacteria entering the body through skin wounds or mucous membranes Person-to-person spread of brucellosis is extremely rare. Infected mothers who are breast-feeding may transmit the infection to their infants.

Sexual transmission has been rarely reported. While uncommon, transmission may also occur via tissue transplantation or blood transfusions (CDC, 2012).

An important aspect of *Brucella* infection is its ability to persist and replicate within phagocytic cells of the reticuloendothelial system as well as in non-phagocytic cells such as trophoblasts. This ability involves a temporary fusion of the *Brucella*-containing vacuole with the lysosome, and subsequent exclusion of the lysosomal proteins. Following this process, the *Brucella*-containing vacuole becomes associated with the endoplasmic reticulum. These endoplasmic reticulum-associated compartments are the niche for intracellular replication of *Brucella* in macrophages, epithelial cell lines and placental trophoblasts. Once inside this compartment, the bacteria can establish chronic infection (Xavier, *et al.,* 2010).

2.4. Immunity to brucellosis

Resistance to intracellular bacterial pathogens such as *Brucella* spp. relies on cell-mediated immunity, which involves activation of the bactericidal mechanisms of antigen-presenting cells (macrophages and dendritic cells) and the subsequent expansion of antigen-specific CD4+ and CD8+ T-cell clones. *Brucella* antigens induce the production of T helper type 1 (Th1) cytokines, and an adequate Th1 immune response is critical for the clearance of *Brucella* infection. Studies on experimental and human brucellosis indicate that interferon-γ (IFNγ) is the principal cytokine active against *Brucella* infection. On the other hand, *Brucella* has evolutionarily developed diverse evasion strategies to avoid the host's innate and adaptive immunity in order to establish an intracellular niche for long-term parasitism. Disturbances of the Th1 response and anergy have been described in patients with chronic brucellosis, and are associated with poor outcome. Accordingly, chronic brucellosis represents a challenge for the study of immune mechanisms against *Brucella* and the development of novel therapeutic or vaccination approaches (Skendros and Boura, 2013).

2.5. Antigenic type

Two different O chains in brucellae occur in the LPS of the brucellae with smooth colonies. These are called A and M, nominally indicating *abortus* and *melitensis* antigens. ('Nominally', because some *abortus* biovars carry the M

antigen and some common *melitensis* biovars the A antigen.) Both O chains have been shown to be homopolymers of 4,6-dideoxy-4-formamido-d-mannopyranose; they differ only in that in the A chain the sugar molecules are always linked 2-1 whereas the M chain has every fifth junction a 3-1 linkage. In routine serology, smooth species of brucellae cross-react almost completely with each other, but not with rough species and vice versa. Monospecific polyclonal sera reacting only to A or M antigens are prepared by cross absorption and monoclonal antibodies specific for A and M antigens are now available, indicating that there is at least one unique epitope on each type of chain (Alton and Forsyth,2008).

2.6. Clinical spectrum of brucellosis

2.6.1 Osteoarticular infection

Bone and joint involvement including arthritis, bursitis, sacrolitis, spondylitis, and steomyelitis is most commonly described complication of brucellosis and is seen most often fallowing bacteremia with *B.melitensis,* arthritis and sacrolitis are associated with acute disease while spondylitis, vertebral osteomyelitis, ostetis and par vertebral abscess are seen more frequently in chronic infection (Koneman, *et al* 2006).

2.6.2 Central nervous system infection

Neurobrucellosis occur in less than 5% of cases and initially present as encephalitis, meningencephalitis.

Meningomyelitis or cerebrllar atoxia, patient have cerebral spinal fluid pleocytosis, elevated protein, low to normal glucose, patient with acute meningitis present with headache, vomiting, fever, visual symptom (Koneman, *et al.,* 2006).

2.6.3 Respiratory tract infection

Pulmonary Brucella infection may result from hematogenouse dissemination and sealing the lungs or by direct inhalation of the organism in aerosols, clinical manifestations of pulmonary brucellosis may include bronchitis, bronchopneumonia, lung abscess, pulmonary nodules hilarlymphadenopathy, empyma and plural effusion (Koneman, *et al.,* 2006).

2.6.4 Cardiovascular infection

Is rare complication, occuring in less than 2% of infected patients. Interestingly Brucella endocarditis main cause of death related to this disease, endocarditis may occure complication septic embolization, mycotic, mysarditis and pericarditis (Koneman, *et al.,* 2006).

2.6.5 Genitourinary tract

Brucella may infect genitourinary tract usually as consequence of systemic infection which they may cause epididymitis, prostatis, orchitis and renal granuloma. Although renal involvement is fairly uncommon, glomerulonephritis and pyelonephritis have been reported.

Brucella cause abortion in infected animals by localization in chorioamniotic membrane of placenta, but there is little evidence support role of these organisms in spontaneous abortion in human, however the organism may rarely be isolated from amniotic fluid and placental tissue of human with brucellosis (Koneman, *et al.,* 2006).

2.6.6 Gastrointestinal/ hepatobiliary tract infection

Gastrointestinal And or hepatosplenic brucellosis occur as manifestation of acute systemic in over 70% of patients with brucellosis. The symptoms are abdominal pain, nausea, vomiting, anorexia, diarrhea or constipation. the longstanding of these symptoms may cause colitis, entrocolitis spontaneous bacterial peritonitis, pancreatitis, choleystitis hepatosplenic abscesses and splenic infarcts.

Liver involvement more extensively with *B. melitensis* and *B.suis* infection. Infection of these two species are associated with formation of caseating hepatic granulomas and micro abscesses, while *B.abortus* tend to produce non caseating granulomas in the liver (Koneman, *et al*., 2006).

2.6.7 Ocular infection

Are unusual, late complication of *Brucella* infection and include optic neuritis, uveitis, kerotitis, enophthalimitis and infection of the lacrimal glands, optic neuritis which associated with loss of vision, fever, temporal headache and retrobulblar pain. Endophthalmitis result from hematogenoces dissemination (Koneman, *et al.,* 2006).

2.7. Diagnosis

The diagnosis of brucellosis is primarily dependent on clinical suspicion allied with the taking of an adequate history of possible exposure - including during travel. Presentation can, however, be highly atypical and focal lesions may present decades after exposure. Unequivocal diagnosis requires isolation of the organism. Blood culture is the method of choice but specimens need to be obtained early in the disease and cultures may need to be incubated for up to four weeks. Even so, failure to grow the organism is common, especially in cases of *B.abortus* infection, and isolation rates of only 20-50% are reported even from experienced laboratories. Modern commercial systems are hampered by the small amount of CO_2 produced during growth. Culture from bone marrow and from presenting foci may be successful. Presumptive identification of cultures from morphology and slide agglutination with specific antiserum should be followed by further work in a reference facility. Molecular techniques for typing are being developed (Alton and Forsyth, 2008).

Most standard media, e.g. blood agar, chocolate agar, trypticase soy agar and serum dextrose agar, are suitable for culturing *Brucella* spp. Some strains may need bovine or equine serum (2%–5%) for growth, which is routinely added to the basal medium. The inoculated agar plates should be incubated at 35°C to 37°C in 5% to 10% CO2. In primary

culture from clinical specimens, it can take several days or even weeks before the punctate, non-pigmented and non-haemolytic colonies that are typical of this fastidious bacterium become visible. Colonies of smooth (S) *Brucella* strains are raised, convex, circular, translucent, and 0.5 mm to 1 mm in diameter.

Colony morphology, as well as virulence, antigenic properties and phage sensitivity of the bacteria, is subject to changes after subcultivation or prolonged culture (more than four days). Thus, smooth Brucellae dissociate to rough (R) forms, which grow in less convex and more opaque colonies with a dull, dry, yellowish-white granular appearance. The recovery rate from clinical specimens can be maximized (Sippel *et al.,*1982).

Identification and characterization of *Brucella* spp

In human brucellosis, detection of the agent is paramount as early onset of antibiotic treatment prevents chronicity and focal complications Brucellae are very small, faintly stained Gram-negative coccobacilli resembling 'fine sand' when viewed under the microscope. Oxidase and urease-positive bacteria must raise the suspicion of *Brucella*, and the viable pathogen has to be handled in a biosafety level 3 laboratory. The diagnostic tool of choice for initial and rapid confirmation of suspicious colonies is the slide agglutination test using undiluted polyvalent *Brucella* antiserum (anti-smooth serum) mixed with a saline suspension of the

bacteria. A direct urease test on positive blood cultures suggestive of *Brucella* spp. (68) or

fluorescence *in situ* hybridisation (FISH) using *Brucella* specific probes (82) can also be used to reduce the time to the presumptive diagnosis of human brucellosis.(Sippel *et al.,* 1982)

2.7.1 Rose Bengal test

The rose Bengal acidified card test antigen in plate agglutination test that has been widely used in Europe and soviet union for clinical diagnosis, epidemiologic investigation , and blood donor screens. reported 100% sensitivity of rose Bengal test for patient with culturally proven brucellosis or serologic evidence of active infection , prefer the rose Bengal test for initial screening and the tube agglutination test for retesting of positive sera . the rose bengal test is marketed in Europe as the brucelloslide- test(Balows *et al.*, 2012).

2.7.2 Indirect Coombs (antihuman globulin) test

The Coombs test is an extension of SAT, used for the detection of incomplete, blocking or non-agglutinating IgG . Those SAT tubes containing serum dilutions and whole B.abortus or B.melitensis cells (as antigens) that were negative after incubation for 24 h are centrifuged, the supernatant decanted and the cell pellet re suspended and washed with phosphate-buffered saline, repeated three times. Standardized antihuman globulin reagent (anti-IgG) is

added to the last pelleting in each test tube. The pellet is re suspended and incubated in a water bath at 37◦C for 24 h. Agglutination can be determined visually, as for SAT, by using an agglutinoscope or a drop on a slide examined under the microscope. The test is good for complicated and chronic cases and misses around 7% of cases compared with ELISA (George, 2010).

2.7.3 Brucella capt (Vircell, Granada, Spain)

This test is based on an immunocapture agglutination technique and in a single step detects non-agglutinating IgG and IgA antibodies, as well as agglutinating antibodies. The test is performed according to the manufacturer's instructions: a specified volume of each serum dilution is added to a microplate with U-shaped wells pre-coated with antihuman immunoglobulin. Then a whole-cell, formaldehyde-killed, colored B.melitensis antigen suspension is added. The plate is incubated for 18–24 h at 37◦C before reading visually. Positive reactions show agglutination over the bottom of the well. Negative reactions present a pellet in the centre of the bottom of the well Brucellacapt offers a valuable alternative to the Coombs test, since it shows similar sensitivity and specificity, similar performance (especially in diagnosing complicated and chronic cases), and most importantly is more rapid and less cumbersome to carry out (George, 2010).

2.7.4. Mercaptoethanol (2ME) test

   The usefulness of the 2ME test in the diagnosis of human brucellosis remains controversial. This test, in which the SAT is performed with addition of 2ME in afinal concentration of 0.005M, is claimed by proponents to spilt disulfide bonds of 19s macroglobulins inhibiting agglutination by IgM antibodyes, without interfering with agglutination by 7s9 (IgG) antibodies. In other study that disappearance of IgG antibodies, as measured by the 2ME test in patient treated for acute brucellosis was prognostic of a successful outcome (Edward and Michael, 1989).

A modified rapid slide agglutination test for the presumptive identification of Brucella canis infection in dogs has been developed. The method required mixing 0.1 ml of canine serum with 0.1 ml of 0.2 M 2-mercaptoethanol solution. Equal volumes (0.05 ml) of the treated serum and the B.canis plate antigen were mixed. Agglutination results were read within 2 min.

Clinical studies showed 100% agreement between this method and the conventional 2-mercaptoethanol tube agglutination test. Excellent correlation was shown between cultural isolation and the modified rapid slide agglutination test, using sera from experimentally infected dog (Badakhsh *et al., 1982*).

2.7.5. Serum agglutination test (SAT)

Although this test was introduced over a century ago, in 1897, it remains a cornerstone of the serodiagnosis of brucellosis. The test is performed in tubes by reacting a known standardized volume and concentration of whole Brucella cell suspension with a standardized volume of doubling serum dilutions, usually ranging from 1:20 to 1:1280. The suspension mixture is incubated in a water bath at 37◦C for 24 h, and agglutination at the bottom of the tubes is examined visually. The highest serum dilution showing more than 50% agglutination is considered the agglutination titre. Commercial cell suspensions could vary in quality and thus each cell lot should be quality controlled with known positive and negative standard sera before accepting it for clinical testing. SAT measures total Brucella antibody (IgG, IgM and IgA) (George, 2010).

2.7.6. Micro agglutination test (MAT)

This is essentially a miniaturized format of SAT performed in micro titer plates (V- or U-bottomed). Its advantage over SAT is that it uses smaller volumes of serum and reagents, and can test multiple samples at the same. The agglutination tests show good performance in diagnosing patients with acute brucellosis. However, the drawback of this test is the high false-negative rates in complicated and chronic cases, are liable to false-positive findings because of cross-reactions, and both are cumbersome to set up (George, 2010).

2.7.7. Enzyme linked Immunoassorbent assay

ELISA tests for total (IgG+IgM+IgA), IgG, and IgM anti-Brucella antibodies, which utilised only commercially available reagents, were used to diagnose human brucellosis. Assays for total antibodies in sera from 22 patients with confirmed acute brucellosis, 1 patient with probable acute brucellosis, and 3 patients with probable chronic brucellosis gave readings that were more than double those found in hundreds of control sera. All sera from patients with acute and chronic brucellosis had significantly elevated IgG levels. Although there were a few acute patients with IgM levels only slightly higher than those of some controls, most patients with acute disease could readily be differentiated from both the non-brucellosis patients and patients with chronic brucellosis by measuring macroglobulin's. Both the IgG and IgM levels in sera from acute patients persisted for at least 8 months. The results of this study show that ELISA is an excellent method for screening large populations for Brucella antibodies and for differentiation between the acute and chronic phases of the disease. (Sippel *et al.*, 1982)

2.7.8. Indirect fluorescent antibody test (IFA)

The test involves fixing (by acetone) a predetermined suspension of whole *B.abortus* or *B. melitensis* cells (obtained from different commercial sources or reference laboratories) on acetone-resistant slides. After the addition of doubling serum dilutions, incubation (30 min at 37∘C) and washing in phosphate-buffered saline, fluorescein-labelled antihuman IgG, IgM or IgA is added to the designated circles on the slide, which is incubated (30 min at 37∘C), repeatedly washed, and dried before being mounted with, e.g. Fluoroprep. The slides are read using a fluorescence microscope to determine the titer that is the highest dilution showing positive fluorescence. Positive and negative control sera should be included in each run. The test is rapid (2–3 h) and shows comparable results to ELISA. However, it is subjective in reading, may fail to detect IgA, and differences in reactions to antigens obtained from different manufacturers have been observed (George, 2010)

2.7.9 Immunochromatographic Lateral flow Assay

This assay is run using a composite strip in a plastic device consisting of a nitrocellulose detection strip and a reagent pad. The former contains Brucella LPS antigen, as a Brucella-specific capture probe, and a reagent control applied in distinct lines. The reagent pad contains dried and stabilised detection reagent consisting of acolloidal gold-conjugated antihuman IgG or IgM. The serum sample is added to a sample well, followed by test liquid.

The result is read based on positive or negative staining after 10–15 min by visual inspection of the antigen and control lines in the test window. These Brucella-specific IgG and IgM lateral flow assays have been advocated for screening/surveillance of patients with brucellosis in endemic areas and as outbreak and field tests. They are simple, rapid, easy to perform and read, with high (>90%) sensitivity and specificity.(George , 2010)

2.7.10 Molecular assays

Advances in molecular-based technology have been utilised for the laboratory diagnosis of human brucellosis. In-house developed conventional polymerase chain reaction (PCR) and real-time PCR (RT-PCR) assays have been attempted for the direct detection of Brucella from clinical specimens, to monitor treatment response, and for the identification, speciation and differentiation of recovered Brucella spp. The sensitivities of these assays in the direct detection of Brucella from clinical specimens have been quite variable, ranging from 50% to 100%, and specificity has varied between 60% and 98%. This variation might be related to different DNA extraction methods, detection formats and limits, and to different types of specimens used. The ribosomal 16S–23S internal transcribed spacer regionseems to constitute a suitable target in clinical specimens and formalin-fixed paraffin-embedded archived tissue, as well as for the speciation of

isolates from culture [56]. Overall, molecular assays have good potential in the investigation of patients with brucellosis. So far, however, further standardisation and optimisation are necessary to achieve consistency and reliability of results before they are incorporated in routine laboratory investigations(George, 2010)

2.8. Brucella Typing Procedure

Once an isolate has been identified as a *Brucella* culture, it may then be subjected to further tests in order to identify its species and biovar. In some cases it may be necessary to determine if the isolate has the characteristics of one of the live vaccine strains of *B. abortus* or *B. melitensis.* In all case, when typing organisms suspected to be *Brucella*, it is essential to include in the test at least the *Brucella* reference strains *B.abortus* 544, *B.melitensis* 16M and *B.suis* 1330 as a check on media and methods. If the strains undergoing characterization are non-smooth and/or are thought to belong to one of the other *Brucella* species, then at least one strain typical of these should be included in the test. It is recommended that the procedure is carried out at a specialist reference centre to further identify the species and biovar level (Stack and MacMillan, 2003).

2.9. Dissociation

Brucella cultures growing *in vitro* may undergo changes in colonial morphology which are accompanied by alterations in antigenic structure, phage susceptibility and virulence. This

process is termed dissociation and is particularly likely to occur when smooth strains are grown in static liquid culture. The variants produced may range from grossly aberrant mucoid or rough forms to others which are transitional between the extreme stages; intermediate or smooth-intermediate forms.

 A variety of methods are available for detecting dissociation:

a) Direct observation using obliquely transmitted light
b) Staining of colonies with crystal violet
c) Agglutination in acriflavine
d) Agglutination by antiserum to rough *Brucella*

B.ovis and B.canis are naturally rough on primary isolation.

2.9.1 Growth requiring CO_2

Some biovars of *B. abortus* and *B. ovis* require CO_2 for growth

2.9.2 Production of hydrogen sulphide

Lead acetate strips are used to identify the production of H_2S during growth. For example, *B. suis* biovar 1 produces large amounts but *B. melitensis* is negative (Stack and MacMillan, 2003)

2.9.3. Urease Test

In general, *B.canis, B.neotomae* and *B.suis* will give a strong urease reaction which turns Christensen's medium and magenta colour in less than 15 minutes. *B.ovis* does not hydrolyze urea, neither do some strains of *B.abortus*, including *B.abortus* 544 the type strain for biovar 1 .

2.9.4. Growth on dye plates

Growth is measured on SDA plates containing basic fuchsin and thionin. All biovars of *B. melitensis* grow in the presence

of fuchsin and thionin althuogh recently, atypical *B.melitensis* biovar 1 strains which are resistant to these dyes have been reported. All biovars of *B. abortus*, except biovar 2 grow in the presence of fuchsin.

2.9.5. Agglutination reactions

Monospecific autisera to smooth and rough forms of *Brucella* are used.

2.9.6. Phage sensitivity

Currently used for culture identification are Tb, Wb, Fz, Bk and R phages. These are propagated at CVL and titrated against appropriate host *Brucella* strains. Lysis of strains by phage forms a distinct and integral part of the routine identification procedure.

2.9.7. Oxidative metabolism

In addition, the tetrazolium reduction assay can be performed to support the above classical biotyping methods if required (Stack and MacMillan, 2003)

2.10. Identification of vaccine strains

Using the above classical techniques, Strain 19 cannot be distinguished from other CO_2 independent strains of *B. abortus* biovar 1. Additional tests can be performed using thionin blue and *meso*-erythritol dyes in conjunction with growth in the presence of penicillin to aid differentiation. Also, *B. melitensis* strain Rev 1 cannot be differentiated from virulent isolates of *B. melitensis* biovar 1 using routine techniques.   Supported tests include using penicillin and

streptomycin to discriminate between the vaccine strain and field isolates (Stack and MacMillan, 2003)

2.11. Treatment

The objective of the present prospective, non comparative, multicenter study was assess the safety and efficacy of gentamicin and doxycycline therapy for human brucellosis. In the first part of the study, a cohort of 17 patients received 100 mg of doxycycline (or 50 mg/kg of body weight per day if the body weight was < 40 kg) orally every 12 h for 45 days (cohort 1). In the second part of the study a subsequent cohort of 35 patients was treated with doxycycline at the same dosage for 30 days (cohort 2). All patients were treated intramuscularly with gentamicin at 240 mg (or 5 mg/kg per day if the body weight was < 50 kg) once daily for the first 7 days. Both cohorts showed a favorable response during therapy, and there were no therapeutic failures. Relapse was noted in 1 (5.9%; 95% confidence interval [95% CI], 0.15 to 28.7%) of the 17 patients in cohort 1 and in 8 (22.9%; 95% CI, 10.4 to 40.1%) of the 35 patients in cohort 2. Nineteen patients (36.5%; 95% CI, 23.6 to 51.0%) had adverse effects, with no differences between cohorts, and no patients had a treatment-limiting adverse effect

The study indicates that the combination of doxycycline for 45 days and gentamicin for 7 days is an effective and well-tolerated therapy for human brucellosis.

The relapse rates obtained with doxycycline treatment for 30 days appear to be higher than those obtained with doxycycline treatment for 45 days

In summary, this study demonstrates that the combination of doxycycline for 45 days and gentamicin for 7 days is an effective and well-tolerated therapy for brucellosis. This combination is relatively inexpensive; gentamicin can be given intramuscularly once daily, and it is suited to outpatient therapy. The combination also appears promising for the treatment of brucellosis in patients with focal disease such as sacrolitis and peripheral arthritis. However, the most convenient duration of doxycycline in the doxycycline-gentamicin regiment requires prospective randomized trials. Such a clinical trial is being performely by our group. (Solera *et al*., 1997)

2.12. Animal vaccine

Brucella abortus is a bacterium that causes brucellosis in cattle. *B.abortus* RB51 is a strain of this bacterium developed specifically for immunization of cattle against brucellosis to allow serological differentiation between naturally infected and vaccinated animals. Accidental human exposure to RB51, though uncommon, has resulted in development of symptoms consistent with brucellosis. Exposures have included needle sticks, eye and wound splashes, and contact with infected material. Other vaccines, such as *Brucella abortus* S19 for cattle and *B.melitensis* Rev-

1 for sheep and goats, can also cause infection in humans (CDC, 2012).

CHAPTER THREE

MATERIALS AND METHODS

3.MATERIALS AND METHODS

3.1. Study Design

This was descriptive cross sectional study.

3.2. Study area and duration

This study was carried out in Kamleen including infected reproductive women diagnosed as brucellosis patient by clinical symptoms and slide agglutination during March-July 2015.

3.3. Study population and sample size

One hundred and fifty samples were collected from all brucellosis suspected   reproductive women in Kamleen

3.4. Ethical consideration

Approval to conduct this study was obtained from the College of Graduate Studies, Sudan University of Science and Technology. Permission was obtained from the patients and verbal their consent was taken.

3.5. Specimens collection

Four ml from venous blood were collected in plain container using sterile disposable syringes left to clot for 15mins, centrifuged at 3000rpm for 5mins and serum was separated and storged at -20ºC till used.

3.6. Laboratory methods:-

Slide agglutination was used as screening test and positive result confirmed by Enzyme Linked Immunoassorbent Assay (ELISA)

3.6.1 slide agglutination test

3.6.1.1principle:

The test depends primarily on the reaction between the *Brucella* antigen and specific antibodies that was assumed to

be present in sera of examined subjects (BIOMED.EYG-CHEM for lab technology).

3.6.1.2 Procedure:

The serum samples and antigen were brought to room temperature.

Twenty microliter of each serum sample were placed on slide. After shaking the antigen bottle, an equal volume of the antigen was placed near each serum spot. They were mixed thoroughly (using a clean glass or plastic rod for each test) to produce a circular or oval zone approximately 2cm in diameter. The mixtures were agitated gently for 4mins at an ambient temperature on rocker, after which the agglutination was read any visible agglutination was considered positive

3.5.2 Enzyme Linked Immunosorbent Assay.

3.5.2.1. The principle

The Enzyme Liked lmmunosorbent Assay(ELISA) is an immunoassay, which is particularly suited to the determination of antibodies in the field of infectious serology. The reaction is based on the specific interaction of antibodies with their corresponding antigen the test strips of the SERION ELISA(Virion\Serion Germany) classic microtiter plate are coated with specific antigens of the pathogen of interest. lf antibodies in the patients serum sample are present, they bind to the fixed antigen. A secondary antibody, which has been conjugated with the enzyme

alkaline phosphatase, detects and binds to the immune complex. The colourless substrate p-nitrophenylphosphate is then converted to the coloured product p-nitro phenol. The signal intensity of this reaction product is proportional to the concentration of the analyte in the sample and is measured photometrical.

3.5.2.2. Procedure

The samples were pre-treated with rheumatoid factor-absorbent (RF-dilution buffer) prior to IgM detection, one part of RF absorbent was added to four part of buffer V2 (protein containing phosphate buffer with Tween 20). 200 µl from RF-absorbent was added to 800 µl of dilution buffer. Patient samples (10µl), were diluted in 1000µl RF- absorbent dilution buffer and incubated for 15mins at room temperature. After dilution and before pipetting into the microtiter plate the samples were mixed thoroughly. Diluted samples ,control and standard sera were pipetted into micro test well (100µl) and incubated for 60 min at 37ºc.

The microtiter plate was well washed manually four times by washing solution (300 µl), then 100 µl conjugate solution anti-human   IgM- polyclonal conjugate (APC) was pipetted into the well , incubated at 37ºc for 30 mins.

The plate was washed four times with wash buffer then100µl substrate solution para- nitro phenylphosphate (pNPP) was pipetted, incubated for 30 min at 37ºc

Stopping solution (NaoH) was Pipetted and Optical density

(OD) was read at 620nm wevelenagth

Interpretation of the result:
Fixed the Cutt-off ranges by multiply the mean value of the measured standard OD with numerical data of quality control certificate
Mean of standard = (0.282+0.283) ÷ 2= 0.2825
OD= 0.502×MW(STD) with upper cut-off
0.502×0.2825=0.142
OD= 0.352×MW(STD) with lower cut-off
0.352×0.2825=0.099
The cutt-off is in between 0.099-0.142
Above the 0.142 was positive   lower than .099 was negative.

3.6 Data analysis
statistical package of social sciences (SPSS version 17.). Computer software was used for data analysis. Significant level were set at (p $\leq$ 0. 05).

___

CHAPTER FOUR
RESULTS

4. RESULTS

Out of the blood samples collected from 150 females, housewives were70 (46.7%), students 55 (36.7%), livestock breeders 17 (11.3%) , Veterinarians 8 (5.3%).(in table 1)

Table (1) **The occupation of screened sample**

Occupation

		Frequency	Percent	Valid Percent	Cumulative Percent
Valid	Housewives	70	46.7	46.7	46.7
	students	55	36.7	36.7	83.3
	Livestock breeders	17	11.3	11.3	94.7
	Veterinarians	8	5.3	5.3	100.0
	Total	150	100.0	100.0	

One hundred of out of 150 (66.6%) showed positive agglutination in screening test.(table 2)

Table 2 **frequency of patients negative and positive sample tested by Slide agglutination test**

slide agglutination

		Frequency	Percent	Valid Percent	Cumulative Percent
Valid	Positive	100	66.7	66.7	66.7
	negative	50	33.3	33.3	100.0
	Total	150	100.0	100.0	

Two sample positive by ELISA for IgM from 92 showed in table3.

Table 3 **frequency of patients negative and positive sample tested by ELISA test**.

ELISA

		Frequency	Percent	Valid Percent	Cumulative Percent
Valid	Positive	2	2.2	2.2	2.2
	Negative	90	97.8	97.8	100.0
	Total	92	100.0	100.0	

All veterinarians were free from anti *Brucella* in the screening test, where the livestock breeders showed 7 (4.7%) positive in screening, 55of (22.7%) of the students were positive. 70 (39.3) of The housewives were positive (table 3).

Table 3. **Distribution of brucellosis among occupations**

occupation			slide agglutination		
			positive	Negative	Total
	Housewives	Count	59	11	70
		% of Total	84.3%	15.7%	46.7%
	students	Count	34	21	55
		% of Total	61.8%	38.2%	36.7%
	livestock	Count	7	10	17
		% of Total	41.2%	58.8%	11.3%
	Veterinarians	Count	0	8	8
		% of Total	0.0%	100%	5.3%
Total		Count	100	50	150
		% of Total	66.7%	33.3%	100.0%

Chi-square=0.00 ; p value(p< 0.05)

In ELISA specific for IgM showed two positive, one from house wives another from livestock breeders(table 4).

Table 4 **Distribution of brucellosis using ELISA according to the occupations**

Occupation

		Frequency	Percent	Valid Percent	Cumulative Percent
Valid	Housewives	54	58.7	58.7	58.7
	Students	31	33.7	33.7	92.4
	Livestock	7	7.6	7.6	100.0
	Total	92	100.0	100.0	

ELISA * occupation Crosstabulation

ELISA		Occupation			Total
		housewives	Students	livestock	
Positive	Count	1	0	1	2
	% of Total	1.1%	.0%	1.1%	2.2%
Negative		53	31	6	90
	% of Total	57.6%	33.7%	6.5%	97.8%
Total	Count	54	31	7	92
	% of Total	58.7%	33.7%	7.6%	100.0%

Chi-Square= 0.168 ; P value (P > 0 .05)

The prevalence of brucellosis according to age was 1.03% in young females (15-28 year), in middle ages females (29-41 year) was 1.88% and negative result in old females age (41-55 year old) table5.

Table 5 **Distribution of brucellosis among age groups using ELISA**

Age	Frequency	Positive slide	percentage	Positive Slide	percentage
15-28	72	1	1.03	41	56.9%
29-41	25	1	1.88	9	36%
42-55	53	0	0.00	50	94.3%
Total	150	2	2.91	100	66.6%

In ELISA test one was positive from symptomatic and another from asymptomatic.

Table 6. **The distribution of brucellosis among symptomatic and** --**asymptomatic**

occupation			disease phenotype		Total
			symptomatic	Asymptomatic	
	Housewives	Count	40	30	70
		% of Total	26.7%	20.0%	46.7%
	Students	Count	20	35	55
		% of Total	13.3%	23.3%	36.7%
	Livestock Breeders	Count	10	7	17
		% of Total	6.7%	4.7%	11.3%
	Veterinarians	Count	3	5	8
		% of Total	2.0%	3.3%	5.3%
Total		Count	73	77	150
		% of Total	48.7%	51.3%	100.0%

Chi-Square=.089; P value (P> 0 .05)

In agglutination test *B.melitensis* more than *B.abortus* in table7.8.

Table 7: frequency of *B.abortus*

B.abortus

		Frequency	Percent	Valid Percent	Cumulative Percent
Valid	1\320	6	4.0	4.0	4.0
	1\160	34	22.7	22.7	26.7
	1\80	32	21.3	21.3	48.0
	1\40	78	52.0	52.0	100.0
	Total	150	100.0	100.0	

Table 8**:** frequency of *B.melitensis*

B.melitensis

		Frequency	Percent	Valid Percent	Cumulative Percent
Valid	1\320	6	4.0	4.0	4.0
	1\160	38	25.3	25.3	29.3
	1\80	43	28.7	28.7	58.0
	1\40	63	42.0	42.0	100.0
	Total	150	100.0	100.0	

B.melitensis * B.abortus Crosstabulation

			B.abortus				
			1\320	1\160	1\80	1\40	Total
B.melitens is	1\320	Count	6	0	0	0	6
		% of Total	4.0%	.0%	.0%	.0%	4.0%
	1\160	Count	0	34	4	0	38
		% of Total	.0%	22.7%	2.7%	.0%	25.3%
	1\80	Count	0	0	28	15	43
		% of Total	.0%	.0%	18.7%	10.0%	28.7%
	1\40	Count	0	0	0	63	63
		% of Total	.0%	.0%	.0%	42.0%	42.0%
Total		Count	6	34	32	78	150
		% of Total	4.0%	22.7%	21.3%	52.0%	100.0%

Chi-Square=0.00 P value(P< 0.05)

CHAPTER FIVE
DISCUSSION

5. DISCUSSION

5.1 Discussion

In this study, it was found higher titration of agglutination for *B. melitensis* than *B. Abortus, B.melitensis* This result agrees with   Hashim, *et al.,* 2007 who stated that all positive human sera were observed with *B. melitensis*, Similarly reported that human is more susceptible to *B. melitensis* than *B. Abortus*. The infection might be due to direct exposure and contact of Housewives, students,

livestock breeders, veterinarians   with animals or consumption of raw milk or milk by-products.

However   veterinarians were free from acute brucellosis, that may be due to the good awareness with brucellosis.

In screening test we found hundred samples from hundred fifty were positive to agglutination test.

All higher agglutination in screening test showed negative results by ELISA this might be due to cross reaction with other microorganisms as *Yersinia interocolitica o:9* , *Escherichia coli o:157* and *salmonella urbana,* some types of *Vibrio ,pasteurella. This stated by Alton,G et al,. 1975.*

The study indicated ELISA test is more accurate than slide agglutination test .

In other hand, two cases were found IgM positive that when tested by ELISA, we considered the negative results as asymptomatic cases, it eas supposed to detect the IgG but we were not able to do that for the kits unavailable and highly expensive. The IgM detected in one of the asymptomatic cases was attributed to less frequent contact with Brucella and or contact with low virulence Brucella. .Deklerk and Anderson,1992.

The Housewives category with old ages and livestock category with middle ages were groups that showed positive agglutination while the young ages from students were negatives for this test.

  This could be due to differences in nutrition and immune status and other infectious disease that cause the higher agglutination as described by Hassan (2013).

5.2. Conclusion:

In conclusion low prevalence of acute brucellosis in reproductive females in different occupation in Kamleen area

were found. The agglutination test revealed 100 case of positive cases out of the 150 samples examined where as ELISA revealed only 2 positive cases *B.melitensis* was the major cause of brucellosis.

Veterinarians were negative for brucellosis, Brucella IgM antibody was found in both symptomatic and asymptomatic patients.

5.3. Recommendation

1. ELISA test is recommended to be adopted in diagnosis of brucellosis.

2. Increase the awareness of the disease is highly need.

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Appendix:

Appendix (1):

Sudan University of science and technology
Collage of post graduate studies
Microbiology department
Questionnaire

Questionnaire no. ....   Date ....

Name .... Occupation ....

Residence ....Age ....

Symptoms ....

History of disease ....

Treatment ....